The Cleavage of Rabbit Immunoglobulin G by Trypsin after Mild Reduction and Aminoethylation

By DAVID GIVOL

Section of Chemical Immunology, The Weizmann Institute of Science, Rehovoth, Israel

(Received 30 May 1967)

It has been suggested by Noelken, Nelson, Buckley & Tanford (1965) that in rabbit IgG* the three fragments (two Fab fragments and one Fc fragment) are arranged as three compact globular entities joined by flexible regions of the heavy chains. Papain digestion (Porter, 1959) splits the molecule into these three fragments, whereas limited cleavage with pepsin (Nisonoff, Wissler, Lipman & Woernley, 1960) or CNBr (Cahnmann, Arnon & Sela, 1965) yields, after reduction, fragments that are similar to Fab fragment. Noelken *et al.* (1965) also suggested that the flexible region includes the disulphide bonds, between the two heavy chains, that could be reduced under very mild conditions (Palmer & Nisonoff, 1964). I now report the cleavage of rabbit IgG into three fragments by hydrolysis with trypsin; presumably cleavage takes place near one of the disulphide bonds in the flexible region. This was achieved after the conversion of the half-cystine residues into aminoethylcysteine residues by mild reduction and aminoethylation.

Rabbit IgG (15mg./ml. in 0.15M-NaCl-0.2M-tris hydrochloride buffer, pH8.2) was reduced with various concentrations of dithiothreitol for 50min.

* The nomenclature for immunoglobulin (IgG) and its fragments is that recommended by the World Health Organization (1964).

at room temperature, and was allowed to react with ethyleneimine or iodoacetamide (final concn. 0.1M) for 30min. The solution was then dialysed for 16hr. against 0.15M-NaCl-0.01M-phosphate buffer, pH7.4, and was subjected to hydrolysis with trypsin (enzyme/IgG weight ratio 1:100) at 37°. The digestion was stopped at various times by the addition of soya-bean trypsin inhibitor (gift from Mrs N. Carmel) in equal weight to the trypsin, and samples were analysed in the analytical ultracentrifuge for the sedimentation pattern. Samples before trypsin digestion were hydrolysed with 6N-HCl to determine the aminoethylcysteine content on the short column (20cm. x 0.9cm., 50°, 40ml./hr.) of the amino acid analyser. Aminoethylcysteine emerges from the column between lysine and histidine and a colour value 91% of that of lysine was used for the calculation (Plapp, Raftery & Cole, 1967). The content of aminoethylcysteine/mole of IgG was calculated on the basis of the arginine content (39moles of arginine/mole of IgG; Fleischman, Porter & Press, 1963). Table 1 summarizes some of the results. It is shown that, after reduction with 5mm-dithiothreitol, trypsin cleaves IgG completely to 3.5s fragments if ethyleneimine was used for alkylation, whereas no cleavage was observed if iodoacetamide was the alkylating reagent. This fragmentation by trypsin

Table 1. *Effect of trypsin on reduced alkylated IgG*

IgG (0.9%) was incubated for 2 hr. at 37° with trypsin. Other experimental details are given in the text.

Reduction	Alkylation	% of 3.5s material*	Aminoethylcysteine content (moles/mole of IgG)
—	—	0	—
—	Ethyleneimine	0	0
0.5mm-Dithiothreitol	Ethyleneimine	20	2.6
1.0mm-Dithiothreitol	Ethyleneimine	60	5.7
2.5mm-Dithiothreitol	Ethyleneimine	80	8.9
5.0mm-Dithiothreitol	Ethyleneimine	100	9.5
7.0mm-Dithiothreitol	Ethyleneimine	100	—
7.0mm-Dithiothreitol	Iodoacetamide	0	—

* From the area of the 3.5s peak in the schlieren pattern obtained on ultracentrifugal analysis of the tryptic digests. When partial cleavage was observed, there was an additional component of 4.8s; further incubation with trypsin for 16 hr. did not change significantly the sedimentation pattern.

is quite rapid, and after 30 min. 50% of the IgG was digested to 3.5s fragments. Digestion for 2 hr. was used as a routine to ensure complete fragmentation. When the tryptic hydrolysis was followed in the pH-stat at pH 8.6, the NaOH uptake after 2 hr. was 2–4 moles/mole of IgG. When the reaction mixture was cooled to 0°, the Fc fragment crystallized in the form of small needles and plates, very similar to those described by Porter (1959) for the papain-produced Fc fragment. The crystals could be dissolved and recrystallized many times by alternate warming (37°) and cooling (0°). The crystalline fragment reacted with goat antiserum to rabbit Fc fragment and not with goat antiserum to the rabbit light chain. It has only serine as the *N*-terminal residue (by the dinitrophenylation technique).

The reaction mixture could be fractionated on a CM-cellulose column under the conditions described by Porter (1959) for the papain fragments of IgG. However, only a very small amount of material (approx. 5%) emerged at the position of piece I whereas most of the Fab fragment emerged approximately at the position of piece II; its separation from Fc fragment was therefore difficult. This could be attributed to the introduction of some positive charges during the aminoethylation. The purified material from the position of piece I or II reacted with goat anti-(Fab fragment) and anti-(light chain) sera but not with anti-(Fc fragment) serum. Its amino acid composition was very similar to the sum of the compositions of Fd fragment and light chain (Fleischman *et al.* 1963). It contained 1.7 methionine, 2.6 aminoethylcysteine and 8.7 half-cystine residues, as calculated on the basis of 28 aspartic acid residues/mol. of Fd fragment plus light chain (Fleischman *et al.* 1963).

The mild conditions of reduction used in these experiments did not impair the antibody activity as measured by precipitation of bovine serum albumin with aminoethylated IgG that contained antibodies to bovine serum albumin. The fragments obtained by trypsin digestion of this preparation of aminoethylated IgG were purified on CM-cellulose, and were tested for inhibition of the precipitin reaction between bovine serum albumin and the intact antibody. The Fc fragment did not show any inhibition, whereas the Fab fragment caused 35%, 83% and 100% inhibition in molar ratios 1:2, 2:4 and 3:5 of fragment to the immune IgG respectively (mol.wt. 50000 was used for the fragment, and mol.wt. 150000 for the IgG).

When the washed immune precipitate (formed at a point near equivalence) of the aminoethylated IgG preparation and bovine serum albumin was digested with trypsin (0.15M-NaCl–0.01M-phosphate buffer, pH 7.4) the precipitate dissolved completely in about 20 min., whereas no such effect

was observed with an immune precipitate formed with untreated antibodies even after 24 hr. of incubation.

On sedimentation of the dissolved precipitate in the ultracentrifuge (after 2 hr. of digestion), two sedimenting components were observed: a fast one with $S_{20,w}$ 10.3s and a slow one with $S_{20,w}$ 3.8s. The pattern was very similar to the one observed after the dissolution by reduction of immune precipitates formed with antibodies previously treated with water-insoluble papain (Cebra, Givol & Katchalski, 1962), and presumably represents a soluble complex of antigen and univalent Fab fragments (10.3s) and free Fc fragments (3.8s).

The occurrence of 3.5s fragments after aminoethylation (which converts cysteine into a lysine analogue), but not after carboxamidomethylation, suggests that the cleavage by trypsin is near an aminoethylcysteine residue. It is clear, however, that more than two aminoethylcysteine residues must be introduced/mol. of IgG to obtain complete fragmentation (Table 1); this is consistent with the observation that reduction and alkylation of an average of one disulphide bond in IgG is not enough to obtain a 100% yield of half-molecules (Palmer & Nisonoff, 1964) or a 100% yield of 3.5s fragments after pepsin digestion (Dixon & Nisonoff, 1964).

The similarity of the fragments obtained by trypsin to the papain-produced fragments indicates that the cleavage is in the same region of the papain cleavage. The new *N*-terminal serine residue in Fc fragment implies a sequence Cys-Ser which is in agreement with sequence studies of this area (J. J. Cebra & R. R. Porter, personal communication) and suggests that cleavage with trypsin takes place at the modified half-cystine residue of a disulphide bond.

I am indebted to Professor Michael Sela for his encouragement. Thanks are due to Mr M. Rotman for his skilled technical assistance and Mr A. Lustig for running the ultracentrifuge.

- Cahnmann, H. J., Arnon, R. & Sela, M. (1965). *J. biol. Chem.* **240**, 262762.
 Cebra, J. J., Givol, D. & Katchalski, E. (1962). *J. biol. Chem.* **237**, 751.
 Dixon, D. J. & Nisonoff, A. (1964). *Biochemistry*, **3**, 1338.
 Fleischman, J. B., Porter, R. R. & Press, E. M. (1963). *Biochem. J.* **88**, 220.
 Nisonoff, A., Wissler, F. C., Lipman, L. N. & Woernley, D. L. (1960). *Arch. Biochem. Biophys.* **89**, 230.
 Noelken, M. E., Nelson, C. A., Buckley, C. E. & Tanford, C. (1965). *J. biol. Chem.* **240**, 218.
 Palmer, J. L. & Nisonoff, A. (1964). *Biochemistry*, **3**, 863.
 Plapp, B. V., Raftery, M. A. & Cole, R. D. (1967). *J. biol. Chem.* **242**, 265.
 Porter, R. R. (1959). *Biochem. J.* **73**, 119.
 World Health Organization (1964). *Bull. World Hlth Org.* **30**, 447.